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Bile acids: a potential role in the pathogenesis of pharyngeal malignancy.

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ABSTRACT

Objective: Gastro-oesophageal reflux disease is thought to be a risk factor for head and neck malignancies. Bile acids are one of the principle components of gastric refluxate and have previously been implicated in the development of oesophageal and bowel malignancies. There is clear evidence that bile acids reflux into the laryngopharynx. Despite this, the carcinogenic properties of bile acids in this area are yet to be fully identified. We therefore investigated the potential role of bile acids in pharyngeal malignancy, through the highly conserved process of epithelial-mesenchymal transition (EMT). EMT occurs in invasion and metastasis and is a central process in the development of epithelial carcinoma.

Design: Translational research study

Methods: Human hypopharyngeal squamous carcinoma FaDu cells were challenged with primary (cholic or chenodeoxycholic) and secondary (deoxycholic or lithocholic) bile acids. EMT relevant proteins TGF-β1 and MMP-9 were measured in the cell culture supernates at 48-hours via ELISA. Cell viability was confirmed >95% via CellTiter-Blue assay.

Results: Significantly greater concentrations of TGF-β1 were measured in the culture supernates of cells treated with cholic acid, deoxycholic acid, chenodeoxycholic acid. MMP-9 levels were increased in deoxycholic acid and lithocolic acid stimulations when compared to control ($p<0.05$).

Conclusion: This is the first demonstration that bile acids induce TGF-β1 and MMP-9 in pharyngeal cells. TGF-β1 is considered a master switch for EMT while MMP-9 is a part of the EMT proteome which degrades basement membranes. This implies a potential role for bile acids in pharyngeal carcinogenesis through the mechanism of EMT and suggests potential novel therapeutic targets.

INTRODUCTION

Globally there are 600,000 new cases and 300,000 deaths from head and neck cancer (HNC) worldwide per year[1]. Despite improvements in treatment modalities, HNC still carries a poor prognosis and has a substantial impact on quality of life[2]. While tobacco smoking, alcohol and high-risk papilloma viruses are well recognized etiologic agents, gastro-oesophageal reflux disease (GORD) is being increasingly implicated as a risk factor in the development of HNC, particularly pharyngeal and laryngeal subsites[3,4].

GORD is highly prevalent in the general population[5]. There is clear evidence that duodeno-gastric contents are refluxed beyond the esophagus in so-called extra-oesophageal or laryngopharyngeal reflux (LPR) episodes[6]. Gastric refluxate contains gastric acid, pepsin and bile acids. Evidence of all three components has been found in upper airway samples[7-9].

Increasing evidence is emerging for the role of pepsin and acidic environments in inducing laryngopharyngeal carcinogenesis[10-14]. The carcinogenic properties of bile acids in this area are yet to be fully identified. This is despite the potent carcinogenic properties of bile acids identified in other digestive subsites, such as gastro-oesophageal[15,16] and colonic[17,18].

We therefore investigated the impact of bile acids on a head and neck squamous cell line, specifically the highly conserved process of epithelial-mesenchymal transition (EMT). EMT is a central process in the development of carcinoma and contributes to cancer invasion and metastasis[19]. Transforming Growth Factor beta 1 (TGF-β1) is the recognized 'master switch' that promotes EMT while Matrix Metalloproteinase 9 (MMP-9) is a part of the EMT proteome which degrades basement membranes. Both TGF-β1 and MMP-9 have been shown to play an important role in EMT in HNC[20 21].

MATERIALS AND METHODS

Cell culture

Human hypopharyngeal squamous cell carcinoma FaDu cells (ATCC, USA) were cultured on collagen coated flasks in Eagle's Minimum Essential Medium supplemented with fetal calf serum 10%, non-essential amino acids 0.1mM, Penicillin/Streptomycin 100U/100µgmL^{-1} and L-

Glutamine 2mM (Sigma, USA), incubated at 37°C in a 5% CO_2 incubator. Medium was changed every 2 – 3 days. Upon near confluence, cells were trypsinised (Sigma, USA), diluted in equal volume medium, centrifuged at 200G for 7 minutes and seeded in a new container.

Bile acid preparation and challenge

The four predominant bile acids in the human digestive tract are Cholic acid (CA), Lithocholic acid (LA), Deoxycholic acid (DA), and Chenodeoxycholic acid (CDA). Each stock bile acid (Sigma, USA) was prepared in methanol. Controls contained methanol alone. Bile acids or methanol controls were diluted in resting medium for all experiments. Resting medium comprised standard culture media excluding fetal calf serum. Cells were placed in resting medium for a minimum of 3 hours prior to bile acid challenges in order to promote the resting phase of the growth cycle.

Enzyme-linked immunosorbent assays (ELISAs)

After 48 hours challenge cell culture supernates were collected and stored in a -80°C freezer until use. Human TGF-β1 and Human MMP-9 DuoSet ELISA kits (R&D, USA) were used according to the manufacturers instructions. Samples were diluted to optimum concentrations and absorbance measured at 450nm with a plate reader (Tecan M200) and compared against a known concentration standard curve to calculate unknown concentrations.

CellTiter-Blue Viability Assay

Cell viability was assessed using CellTiter-Blue viability assay (Promega, USA). After the 48-hour challenge period, the supernate was removed and a CellTiter-Blue reagent was added to the cells for 3 hours. No-cell controls were prepared in triplicate to determine the background absorbance. Dead cell controls were also set up in triplicate by adding 100% methanol to cells. Live unchallenged cell controls were also set up in triplicate. Fluorescence excitation and emission ratio was measured at 560nm and 590nm respectively. Percentage viability was calculated from fluorescence of challenged cells against controls.

Statistical Analysis

Data from each experiment compromised 4 biological replicates, each with 2 technical replicates. Analysis was performed on Prism 5 (GraphPad, CA, USA) using Mann-Whitney U tests. Comparison was made between each experimental condition and control results. Data was expressed as mean ± the standard error of the mean (SEM). Significance was taken as $p<0.05$.

RESULTS

We demonstrated a significant ($p<0.05$, $n=4$) increase in the expression of TGF-β1 (via ELISA of cell culture supernates) with exposure to concentrations of CDA 100μM, CA 600μM and DA 100μM, 75μM compared to control samples. LA at concentrations up to 20μM did not produce a significant increase in TGF-β1 levels (*figure 1*). MMP-9 ELISA of cell supernate showed a significant ($p<0.05$, $n=4$) increase in MMP-9 in challenged human FaDu cells compared to controls at concentrations of DA 100μM and LA 100μM. CA at concentrations up to 600μM and CDA at concentrations up to 100μM produced no significant increase in MMP-9

concentrations compared to controls (*figure 2*). Cell viability was confirmed at >95% in all experimental conditions by CellTiter-blue viability assay.

DISCUSSION

Synopsis of key findings

We have demonstrated for the first time a potential link between bile acids and up regulation of proteins implicated in EMT and cancer. We investigated proteins associated with EMT that are recognized to have a substantial role in the development of epithelial tumors and are of significant relevance in HNC[19-21]. TGF-β1 is considered the 'master switch' for EMT and can induce changes in epithelial architecture and phenotype accompanied by an EMT signature proteome, ultimately resulting in the epithelial cells transitioning to a mesenchymal phenotype[21]. MMP-9 is a type IV collagenase and is a potent basement membrane degrading enzyme which has been closely tied to epithelial cells being able to invade into local structures[21].

Significantly greater concentrations of TGF-β1 were measured in the culture supernates of cells treated with cholic acid (CA), deoxycholic acid (DA), chenodeoxycholic acid (CDA), and of MMP-9 in the cell culture supernates of cells challenged with deoxycholic acid (DA) and lithocholic acid (LA), when compared to controls ($p<0.05$). We used concentrations of the differing bile acids up to the maximal concentration that did not induce high cell death based on our optimization work. CellTiter-blue viability assay confirmed >95% cell viability in all conditions, hence release of these EMT markers was from cell expression and not cell death. The variable role of the different bile acids on EMT markers was a surprising finding. TGF-β1 is considered an earlier EMT marker and may promote MMP-9 production[22]. Therefore as DA was

the most potent TGF-β1 stimulator it could be hypothesized that this lead to downstream production of MMP-9. The finding that LA only produced significant increased amounts of MMP-9, and not TGF-β1, requires further investigation of alternative EMT pathways. E-cadherin and Fibronectin are then next two most relevant EMT markers and future study of their expression would be of benefit.

Strengths and weaknesses of the study

A limitation of this study is absence of evidence quantifying the concentrations of each bile acid in the laryngopharynx during or after reflux events. Therefore extrapolation of these current results to the *in vivo* environment is difficult. In humans, CA, CDA and DA are predominantly found in the digestive tract, with only small amounts of LA present (<10%)[23]. The total concentrations of bile acids in gastric juice, derived from the stomach, are quoted as between 10 – 10,000μM[24]. We used a range of between 15 - 600μM for each individual bile acid, so are well within the physiological range expected in gastric refluxate. However, further investigation of the dose response relationship over a broader range of concentrations would be of interest in future studies. The use of a cell line carries numerous limitations and further studies in primary cells is now required. The FaDu hypopharyngeal squamous cell line is however a well established and characterized cell line in reflux and head and neck cancer research[14].

Comparisons with other studies

There is a large body of evidence demonstrating an association between GERD and oesophageal malignancy[25]. The relationship between GERD and head and neck malignancies is less clearly established. A recent meta-analysis by Zhang et al[3] summarized the conflicting findings of recent

population based studies. They highlighted the difficulty in demonstrating an association due to the substantial confounding smoking and alcohol history in the cancer groups.

Several *in vitro* studies have demonstrated a potential role of reflux constituents, pepsin and acid, in prompting laryngopharyngeal carcinogenesis[10-14]. However, Galli et al[26] in a series of 40 achlorhydric gastrectomised patients demonstrated that 15% developed premalignant or malignant laryngeal tumours compared to 2.5% in a control group of dyspeptic patients, indicating a potentially substantial role of bile acids in laryngopharyngeal carcinogenesis. Further to this there is an increasing body of *in vitro* evidence demonstrating a potential link between bile acids and other digestive subsites malignancies, such as gastro-oesophageal[15,16] and colonic[17,18].

We could identify only two other studies, which investigated cellular mechanistic relationships between bile acids and head and neck squamous cells. Sung et al[27] demonstrated increased expresssion of Cyclo-Oxygenase-2 (COX-2) by pharyngeal cells after challenge with CDA. In a more recent study, Sasaki et al[28] demonstrated increased expression of nuclear factor-kappaB (NF-κB) by hypopharyngeal cells after challenge with a cocktail of bile acids. Our study has however, for the first time, been able to demonstrate differing potencies and mechanistic relationships between each of the four main human bile acids on EMT pathways in head and neck squamous cells.

Clinical applicability of the study

This is a preliminary *in vitro* study, which demonstrates a potentially important link between bile acids found in duodeno-gastric refluxate and HNC.

The importance in identifying gastro-duodenal refluxate as a potential co-factor in HNC is the potential for preventative interventions, such as counteracting reflux in individuals at high-risk of HNC. The other essential consideration of bile acids as a specific causative agent in HNC is the need to move away from acid neutralization agents alone. Agents such as proton pump inhibitors do not reduce biliary secretions or impede bile acid function. Other agents such as alginates may have an effect in physically blocking biliary reflux into the laryngopharynx.

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Legends to Illustrations

Figure 1 – Elevation of TGF-β1 in cell culture supernates after 48hr challenge with bile acids: FaDu cells were challenged with bile acids, after 48 hours TGF-β1 concentrations were measured in the cell culture supernates (n=4), *$p<0.05$. CellTiter-Blue viability assay showed >95% cell viability in each experimental condition.

Figure 2 – Effect of bile acids on MMP-9 concentration in cell culture supernates of FaDu cells: FaDu cells were challenged with bile acids, after 48 hours MMP-9 concentrations were measured in the cell culture supernates (n=4), *p<0.05. CellTiter-Blue viability assay showed >95% cell viability in each experimental condition.